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(71) Applicant:
TANABE SEIYAKU CO., LTD.
Chuo-ku, Osaka-shi, Osaka 541-8505 (JP)

(72) Inventors:
- **TANIGUCHI, Tomoyasu**
  Toda-shi, Saitama 335-0023 (JP)
- **MIZUKAMI, Junko**
  Ootsu-shi, Shiga 520-0242 (JP)

(74) Representative:
Müller-Boré & Partner
Patentanwälte
Grafinger Strasse 2
81671 München (DE)

(54) METHODS FOR IDENTIFYING OR SCREENING AGONISTS FOR AND ANTAGONISTS TO PPAR

(57) A method for identifying or screening an agonist for or antagonist to a peroxisome proliferator activated receptor (PPAR) which comprises allowing a test cell and a substance to be tested to coexist, and detecting a change in a ligand-dependent interaction between the PPAR and a coactivator in the test cells due to the substance to be tested by measuring the expression of a reporter gene as an index.

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Description

TECHNICAL FIELD

[0001] This invention relates to a novel method for identifying or screening an agonist for and/or antagonist to peroxisome proliferator activated receptor (PPAR).

BACKGROUND ART

[0002] Peroxisome, an organelle found in the cells of animals and plants, contains a group of enzymes participating in the lipometabolism and absorption of lipids such as cholesterol. An increase in peroxisome is also induced by diet or physiological factors. It is known that a group of chemicals diversified in structure including antilipemic (fibrates), insecticides and plasticizers such as phthalic acids when they are administered dramatically increase the size and number of peroxisome in liver and kidney and at the same time elevate the ability of metabolizing fatty acids in peroxisome through intermediary of an increase in the expression of enzymes necessary for the β-oxidation cycle. Hence, they are called peroxisome proliferator. Among studies on the mechanism of such a peroxisome proliferation, a nuclear receptor that is activated by the group of chemicals has been identified and named peroxisome proliferator activated receptor (PPAR).

[0003] From its structure, etc., PPAR is considered to be a member of nuclear receptor (nuclear hormone receptor) super family. Like other nuclear receptors, it is activated by its binding to a ligand, and its binding to a response sequence (PPRE: peroxisome proliferator response element) existing upstream of a target gene domain activates transcription of the target gene. PPAR is known to form a heterodimer with a retinoid X receptor (RXR) and binds to PPRE in the form of the heterodimer. Also, like other nuclear receptors, PPAR is considered to have the interaction with a group of transcription coactivators (coactivators) in order to exhibit its transcription activation activity.

[0004] Hitherto, three kinds of PPAR subtypes called PPARα, PPARδ (or NUC-1, PPARβ, FAAR) and PPARγ have been identified and their genes (cDNA) have been cloned (Lemberger et al., Annu. Rev. Cell. Dev. Biol., vol. 12, pp. 335-363, 1996). Of the three kinds, PPARγ is expressed particularly in an adipose tissue and considered to be a factor that closely participates in differentiation of adipocytes (Tontonoz et al., Genes and Development, vol. 8, pp. 1224-1234, 1994; Tontonoz et al., Cell, vol. 79, pp. 1147-1156, 1994).

[0005] On the other hand, various thiazolidinedione derivatives show hypoglycemic effect in a model animal of non-insulin-dependent diabetes mellitus (NIDDM) and are expected as a NIDDM remedy having an insulin resistance releasing effect. These thiazolidinedione derivatives act as ligands to PPARγ and specifically activate PPARγ, which has been discovered in recent studies (Lehmann et al., Journal of Biological Chemistry, vol. 270, pp. 12953-12956, 1995). Since a strong correlation is observed between such a PPARγ activation ability of thiazolidinedione derivatives and the hypoglycemic effect in a hereditary obese mouse, PPARγ is considered to be a target molecule of the pharmaceutical effect of the thiazolidinedione derivatives (Willson et al., Journal of Medicinal Chemistry, vol. 39, pp. 665-668, 1996). This also relates to the fact that an adipose tissue where PPARγ is specifically expressed is an organ that plays an important role in maintaining energy balance. From these findings, a compound specifically acting as an agonist for PPARγ is considered to be very useful as a remedy for diabetes mellitus.

[0006] However, to date, those methods known as screening methods for PPAR acting agents each involve the problems that operation is complicated and simultaneous treatment of multiple samples is difficult.

[0007] For example, there has been known a method for examining PPAR activation ability of a sample using animal cells having introduced therein reporter plasmid containing a reporter gene linked to a PPAR expression vector and a PPAR response element (PPRE), with using as an index a change in the amount of expression of a reporter gene in the cells (WO 96/22884, Tontonoz et al., Genes and Development, vol. 8, pp. 1224-1234, 1994). As its improved method, there has been known a method using animal cells having introduced therein vector for expressing fused protein in which the DNA binding domain of GAL4, i.e., the transcription factor of yeast and the ligand binding domain of PPAR linked together, along with having introduced a reporter plasmid containing a reporter gene linked to the response element of GAL4 (GAL4 binding element) (WO 96/33724,-Lehmann et al., Journal of Biological Chemistry, vol. 270, pp. 12953-12956, 1995; Willson et al., Journal of Medicinal Chemistry, vol. 39, pp. 665-668, 1996). In these methods, an extrinsic gene is introduced into animal cells. Upon the introduction of gene, it is sometimes the case that the integration of a gene into a chromosome has taken place, the gene is influenced by the site where the gene is integrated. Therefore, it is necessary to use a transformed cell in which gene is not influenced by the chromosome. To acquire such a transformed animal cell and express an extrinsic gene stably are accompanied by technical difficulties. Since coactivators, RXR, etc. derived from host animal are considered to participate in the activation of transcription in these methods, there is the possibility that the action of the test substance to PPAR alone cannot be detected surely.

[0008] As a method for directly detecting the binding between PPAR and a ligand without using any animal cell or reporter gene, there has been known a method for examining binding and antagonism between a fused protein com-

prising the ligand binding domain of PPAR and glutathione-S-transferase (GST) and a test compound labeled with a radioisotope (Willson et al., Journal of Medicinal Chemistry, vol. 39, pp. 665-668, 1996; Buckle et al., Bioorganic & Medical Chemistry Letters, vol. 6, pp. 2121-2126, 1996). Recently, it has been elucidated that like other nuclear receptor RXR, etc., PPAR interacts with SRC-1, one of coactivators, ligand-dependently. Based on this finding, Krey et al. reported a method for detecting the action of a test compound as a ligand using a fused protein comprising the ligand binding domain of PPAR and glutathione-S-transferase (GST) and SRC-1 labeled with a radioisotope (Krey et al., Molecular Endocrinology, Vol. 11, pp. 779-791, 1997). However, these methods each use a label of radioisotope and therefore it is accompanied by a danger and has a limitation in treating power since preparation of a labeled compound or coactivator on a large scale is difficult.

[0009] As described above, upon screening PPAR acting agents, a screening method which is simple, high precision, and efficient has been desired.

[0010] An object of this invention is to provide a novel method for identifying and screening an agonist and/or antagonist to peroxisome proliferator activated receptor (PPAR).

[0011] The present inventors have uniquely found that in addition to SRC-1, one of the coactivators, that is already known to interact with PPAR, CBP (CREB-binding protein) interacts with PPAR ligand-dependently and identified the binding domain of the coactivator to PPAR. Further, based on these findings, they have completed a method for identifying or screening a novel PPAR acting agent that detects a ligand-dependent interaction between PPAR and a coactivator using a two-hybrid system of yeast.

DISCLOSURE OF THE INVENTION

[0012] This invention relates to a method for identifying or screening an agonist for or antagonist to a peroxisome proliferator-activated receptor (PPAR), which comprises allowing a test cell and a substance to be tested to coexist, and detecting a change in a ligand-dependent interaction between the PPAR and a coactivator in the test cells due to the substance to be tested by measuring the expression of a reporter gene as an index.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013]

Fig. 1 is a schematic diagram illustrating the constitutions of used plasmids pGBT9-PPARγ2 and pGAD424-CBP, respectively;
Fig. 2 is a diagram illustrating ligand-dependent interaction between PPARγ and CBP; and
Fig. 3 is a diagram illustrating action of T-174 to the interaction between PPARγ and CBP.

BEST MODE FOR CARRYING OUT THE INVENTION

[0014] In this invention, a ligand-dependent interaction between PPAR and a coactivator in the test cells is detected. PPAR changes its conformation into an activated type by binding to a ligand and the interaction with a coactivator takes place. That is, the ligand-dependent interaction is the binding of PPAR with the coactivator promoted in the presence of a ligand of PPAR.

[0015] As PPAR, subtypes such as PPARα, PPARδ (or NUC-1, PPARβ, FAAR) and PPARγ are known. In this invention, any one of these subtypes can be used. Among these, PPARγ is a target molecule of thiazolindinedione derivatives having an antidiabetic effect. A method for identifying or screening a specifically acting agent therefor is useful in research and development of a remedy for diabetes mellitus.

[0016] PPAR may be derived from any species so far as it is identified as the same molecular species and exhibits its function in the organism as a nuclear receptor. For example, it includes those derived from mammalians such as human, mouse, rat, hamster, etc., and in addition those derived from clawed toad (Xenopus laevis). From the point of view of utilizing research and development of a remedy for humans, it is preferred to use human-derived one out of these.

[0017] The gene sequences and amino acid sequences of PPARα (Dreyer et al., Cell, vol. 68, pp. 879-887, 1992, Green et al., Nature, vol. 347, pp. 645-650, 1990, Goettlicher et al., Proc. Natl. Acad. Sci. USA, vol. 89, pp. 4653-4657, 1992), PPARδ (or NUC-1, PPARβ, FFAR) (Dreyer et al., Cell, vol. 68, pp. 879-887, 1992, Kliewer et al., Proc. Natl. Acad. Sci. USA, vol. 91, pp. 7355-7359, 1994, Amri et al., Journal of Biological Chemistry, vol. 270, pp. 2367-2371, 1995, Xing et al., Biochem. Biophys. Res. Commun., vol. 217, pp. 1015-1025, 1995) and PPARγ (Dreyer et al., Cell, vol. 68, p. 879-887, 1992, Zhu et al., Journal of Biological Chemistry, vol. 268, pp. 26817-26820, 1993, Kliewer et al., Proc. Natl. Acad. Sci., USA, vol. 91, pp. 7355-7359, 1994, Mukherjee et al., Journal of Biological Chemistry, vol. 272, pp. 8071-8076, 1997, Elbrecht et al., Biochem. Biophys. Res. Commun., vol. 224, pp. 431-437, 1996, Chem et al., Biochem. Biophys.

Res. Commun., vol. 196, pp. 671-677, 1993, Tontonoz et al., Genes & Development, vol. 8, pp. 1224-1234, 1994, Aperlo et al., Gene, vol. 162, pp. 297-302, 1995) have already been reported. PPARγ includes two kinds of isoforms, PPARγ1 and PPARγ2. PPARγ1 as compared with PPARγ2 is deleted of 30 amino acids on the N-terminal side but the other amino acid sequence is quite the same. Each is expressed in an adipose tissue.

[0018] Among the reports, presuming from the homology with other nuclear receptor, etc., the ligand binding domain (LBD) of PPAR is considered to corresponds to the domain including about No. 167 to 468 amino acids from the N-terminal side in the case of PPARα, to the domain including about No. 138 to 440 amino acids from the N-terminal side in the case of PPARδ, and to the domain including about No. 174 to 475 amino acids from the N-terminal side.

[0019] To detect the interaction between PPAR and coactivator, a polypeptide including at least the ligand binding domain may be used. Cut and use of a polypeptide including the ligand binding domain of PPAR can exclude nonspecific interaction and hence are preferred.

[0020] The coactivator used in this invention may be any one so far as it interacts with PPAR ligand-dependently, that is, the interaction with PPAR in the presence of a ligand of PPAR is promoted. The coactivator which is considered to interact with nuclear receptor includes, for example, CBP, SRC-1, RIP140 (Cavailles et al., EMBO Journal, vol. 14, pp. 3741-3751, 1995), TIF1 (Douarin et al., EMBO Journal, vol. 14, pp. 2020-2033, 1995, Vom Baur et al., EMBO Journal, vol. 15, pp. 110-124, 1996), TIF2 (Voegel et al., EMBO Journal, vol. 15, pp. 3667-3675, 1996), SUG1 (Vom Baur et al., EMBO Journal, vol. 15, pp. 110-124, 1996), P300 (Chakravarti et al., Nature, vol. 383, pp. 99-103, 1996), etc. These are expected to interact also with PPAR ligand-dependently.

[0021] Among these, CBP and SRC-1, as shown in Examples in the present specification later on and in the report by Krey et al., have been confirmed to interact with PPAR and can be used advantageously in this invention.

[0022] CBP (CREB-binding protein) is a protein that has been originally identified as a coactivator of transcription factor CREB (cAMP-regulated enhancer binding protein) that binds to CRE (cAMP-regulated enhancer) and both gene and amino acid sequence thereof have been known (Chrivia et al. Nature, vol. 365, pp. 855-859, 1993; Kwok et al., Nature, vol. 370, pp. 223-226). Recently, it has been revealed that CBP binds not only to CREB but also to a nuclear receptor in the presence of a ligand to serve as a coactivator and that the N-terminal moiety of CBP participates in the interaction with the nuclear receptor (Kamei et al., Cell, vol. 85, pp. 403-414, 1995). That the N-terminal moiety of CBP interacts also with PPARγ ligand-dependently was found uniquely by the present inventors.

[0023] SRC-1 is known to interact with nuclear receptors such as glucocorticoid receptor, estrogen receptor, thyroid hormone receptor and retinoid X receptor (RXR) ligand-dependently and serves as a coactivator. Its gene and amino acid sequence are also known (Onate et al., Science, vol. 270, pp. 1354-1357, 1995). In the Krey et al. report (Molecular Endocrinology, vol. 11, pp. 779-791, 1997), the experiment using the ligand binding domain of clawed toad (Xenopus laevis)-derived PPAR and RI-labeled SRC-1 indicated that PPAR also interacts with SRC-1 ligand-dependently.

[0024] Upon detecting the ligand-dependent interaction with PPAR, the whole coactivator may be used, besides, a polypeptide that contains at least PPAR binding domain (the domain that participates in binding to PPAR) may be used. Coactivators generally have large molecular weights and use of the whole sometimes result in difficulty of expression of protein and it is preferred that appropriate domain be selected and used from this point of view.

[0025] The PPAR binding domain (domain participating in binding to PPAR) of a coactivator can be guessed from information on the position of its binding domain with a nuclear receptor if such an information has been reported. Also, using a system for detecting protein-protein interaction (for example, two-hybrid system of yeast), presence or absence of the interaction of a certain domain with PPAR may be examined and selection of a proper domain may be made. In the case where the coactivator is CBP, then PPAR binding domain exists near the N-terminal moiety (domain including about No. 1 to 450 amino acids).

[0026] In the present invention, the ligand-dependent interaction between PPAR and coactivator is detected in test cells using the expression of a reporter gene as an index and measurement was made of a change in the interaction due to the substance to be tested.

[0027] Noticing the interaction between PPAR and coactivator, the transcription activation effect of PPAR *per se* is not detected, so that various factors inherent to mammals participating in the expression of transcription activation ability of PPAR do not have to be present. Therefore, there is no need to use mammalian cells as test cells. Cells may be any one so far as they are eucaryotic cells. For example, there may be mentioned yeast cells, insect cells, mammalian cells, etc. Among these, yeast cells are advantageous in that their cultivation is easy and can be performed quickly and that application of genetic recombination technique such as introduction of extrinsic genes is easy. As yeast cells, there can be used cell lines of microbes belonging to the genera *Saccharomyces*, *Schizosaccharomyces*, etc., such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, etc.

[0028] As the test cells, usually those that contain extrinsic PPAR and coactivators may be used. Use of cells containing no intrinsic PPAR or coactivators interacting therewith is preferred since the influence due to intrinsic elements can be excluded.

[0029] The change in the interaction between PPAR and coactivator due to the substance to be tested can be efficiently measured by a method utilizing a two-hybrid system.

[0030] The two-hybrid system is a method for detecting protein-protein interaction using the expression of a reporter gene as a marker (U.S. Patent No. 5,283,173 and Proc. Natl. Acad. Sci., USA, vol. 88, pp. 9578-9582, 1991). Many transcription factors can be divided into two domains having different functions, that is, a DNA binding domain and a transcriptional activation domain. In the two-hybrid system, for example, to examine the interaction between the two proteins X and Y, two kinds of fused protein, that is, a fused protein composed of the DNA binding domain of a transcription factor and X, and a fused protein composed of the transcriptional activation domain of a transcription factor and Y are simultaneously expressed in yeast cells. When the proteins X and Y interact with each other, the two kinds of fused proteins form by combination a transcription complex exhibiting a single function as a whole. This transcription complex combines with a response element (the site of DNA to which a transcription factor is bound specifically) in the nuclei of cells and activates transcription of a reporter gene positioned downstream. Thus, the interaction between the two proteins can be detected by the expression of the reporter gene (for example, the enzyme activity of gene products).

[0031] The two-hybrid system can usually be used in the identification of unknown proteins that interact with a specific protein and generally used in qualitative evaluation of protein-protein interaction. The present inventors utilized this system, and thus, completed a method which can quantitatively measure the ligand-dependent interaction between PPAR and a coactivator, and can be applied to the identification or screening of antagonist/agonist for receptors, in which quantitative evaluation is indispensable.

[0032] As one embodiment of the present invention, there may be mentioned a method for identifying an agonist for or an antagonist to PPAR, comprising: using test cells containing

(i) a first fused gene coding for a first fused protein comprising at least ligand binding domain of PPAR and a first domain of a transcription factor, wherein the first domain of said transcription factor being a DNA binding domain or a transcriptional activation domain;

(ii) a second fused gene coding for a second fused protein comprising at least PPAR binding domain of a coactivator which interacts with the PPAR and a second domain of the transcription factor, wherein the second domain of said transcription factor is a transcriptional activation domain when the first domain of the transcription factor is a DNA binding domain or is a DNA binding domain when the first domain of the transcription factor is a transcriptional activation domain, and

(iii) a response element to which the DNA binding domain of said transcription factor can bind and a reporter gene linked thereto,

making the test cells coexist with a substance to be tested, and detecting, by measuring the expression of a reporter gene as an index, a change in the ligand-dependent interaction between the peroxisome proliferator-activated receptor (PPAR) and a coactivator in the test cells occurring due to the substance to be tested.

[0033] In this embodiment, the transcription factor used for detecting the interaction between the PPAR and coactivator is not limited particularly so long as it is a transcription factor (other than PPAR) of eucaryotic organism that can exhibit the function of transcriptional activation in cells. However, it is preferred to use a transcription factor derived from yeast from the viewpoint that it does not need the coactivator, etc. derived from mammalian cells to function and it independently exhibits transcriptional activation ability efficiently in yeast cells.

[0034] Such a transcription factor includes yeast GAL4 protein (Keegan et al., Science, vol. 231, pp. 699-704, 1986, Ma et al., Cell, vol. 48, pp. 847-853, 1987), GCN4 protein (Hope et al., Cell, vol. 46, pp. 885-894, 1986), ADR1 protein (Thukral et al., Molecular and Cellular Biology, vol. 9, pp. 2360-2369, 1989), etc.

[0035] The DNA binding domain of the transcription factor may be those having a DNA binding ability to the response element but alone having no transcriptional activation ability. Also, the transcriptional activation domain of the transcription factor may be those having a transcriptional activation ability but alone having no DNA binding ability to the response element.

[0036] The DNA binding domain and transcriptional activation domain of a transcription factor, in the case of, for example, GAL4, are known to be present on the N-terminal side (a domain including about No. 1 to 147 amino acids) and C-terminal side (the domain including about No. 768 to 881 amino acids), respectively. In the case of GCN4, they are known to be present on the C-terminal side (the domain including about No. 228 to 265 amino acids) and N-terminal side (the domain including about No. 107 to 125 amino acids), respectively. In the case of ADR1, they are known to be present on the N-terminal side (the domain including about No. 76 to 172 amino acids) and the C-terminal side (the domain including about No. 250 to 1323 amino acids), respectively.

[0037] As the response element, a response element corresponding to a transcription factor may be used and DNA sequences to which the DNA binding domain of the transcription factor can bind are used. The response element corresponding to a transcription factor generally exists in a domain upstream of the gene whose transcriptional activity is controlled by the transcription factor, so that such a domain may be cut out for use. If its sequence is known, corresponding oligonucleotide may be synthesized by chemical synthesis and used.

[0038] For example, in case of GAL4 is used as a transcription factor, GAL4-specific DNA sequence called UASg (upstream activation site of galactose genes) may be used as the response element. UASg is contained in the domain upstream of galactose metabolism genes such as the GAL1 gene, etc., so that these domains may be used. Alternatively, a nucleotide sequence corresponding to UASg may be chemically synthesized and used.

[0039] The reporter gene positioned downstream of the response element is not limited particularly so far as it is a commonly used one and it is preferred to use the gene of an enzyme which is stable and allows easy quantitative measurement of its activity, etc. Such a reporter gene includes, for example, β-galactosidase gene (lacZ) derived from E. coli, chloramphenicol acetyltransferase gene (CAT) derived from bacterial transposone, luciferase gene (Luc) derived from a firefly, etc. Among these, E. coli-derived β-galactosidase gene (lacZ) is preferable since it can be readily measured with visible light using a coloring substrate. The reporter gene may be a gene having an original promoter of the gene, or besides, a gene of which promoter part is replaced with one derived from of another gene may be used. The reporter gene may be enough if it is operatively linked downstream of the response element.

[0040] The first fused protein contains the ligand binding domain of PPAR and the first domain of the transcription factor (DNA binding domain or transcriptional activation domain) and the second fused protein contains the PPAR binding domain of a coactivator and the second domain of transcription factor (transcriptional activation domain or DNA binding domain). The two kinds of domains constituting the fused protein may be each arranged in the upstream domain. The fused protein may have additional construction or deletion or substitution of sequence within the range that necessary functions are not damaged.

[0041] The first and second domains of the transcription factor must be integrated before they can bind to the response element and play the function of activating gene transcription. For this purpose, when the first domain is a DNA binding domain, the second domain must be a transcriptional activation domain. When the first domain is a transcriptional activation domain, the second domain must be a DNA binding domain. The first and second domains do not necessarily be derived from the same transcription factor but may be derived from different transcription factors.

[0042] The fused genes coding for the first and second fused proteins may be designed and constructed by using a usual genetic recombination technique. As for the DNA coding for the ligand binding domain of PPAR, PPAR binding domain of a coactivator, DNA binding domain of a transcription factor and transcriptional activation domain of a transcription factor constituting the first and second fused proteins, cDNA may be isolated from cDNA library by, for example, screening, etc., using PCR (Polymerase Chain Reaction) or a synthetic probe which uses a primer or probe designed and synthesized based on the information on the known amino acid sequence or nucleotide sequence. DNAs coding for the respective domains are linked and the resulting material is linked downstream of a suitable promoter to construct a fused gene. To each domain or DNA coding this, it may be introduced addition, deletion, substitution of sequence within the range where necessary functions are not damaged.

[0043] The resulting first and second fused genes may be incorporated into a suitable vector plasmid and introduced into host cells in the form of a plasmid. The first and second fused genes may be constructed so as to be contained on the same plasmid or on separate plasmids.

[0044] The response element and the reporter gene linked thereto may also be designed, constructed using usual genetic recombination technique and the construction is incorporated into the vector plasmid, and the resulting recombinant plasmid may be introduced into host cells. Alternatively, cells in which such a construction is incorporated in chromosomal DNA may be acquired and used.

[0045] Test cells including all the constitution may be acquired, for example, by introducing one or more plasmids containing the first and second fused genes into host cells in which a response element along with a reporter gene linked thereto are introduced into the chromosomal DNA of the host cells.

[0046] The thus obtained test cells are cultivated, for example, in the presence of a substance to be tested, and an interaction between PPAR and a coactivator is detected and measured by the expression of the reporter gene. When the substance to be tested binds to PPAR and an interaction with the coactivator occurs depending on the binding, an increase in the reporter activity is observed. Such a substance to be tested can be identified as an agonist for PPAR. For example, when the substance to be tested binds to PPAR but does not promote the interaction with the coactivator, addition of it together with true ligand or the drug identified as an agonist, a decrease in the reporter activity expressed by the true ligand or agonist is observed. Such a substance to be tested is identified as an antagonist to PPAR.

[0047] Of the invention, as another embodiment of the method in which the ligand-dependent interaction with CBP is detected and the effect of a substance to be tested is measured with respect to said interaction, there is, for example, a method in which the ligand-dependent interaction between PPAR and CBP is measured directly. In this method, for example, CBP or its PPAR binding domain labeled with RI, etc. is used and the binding with a fused protein composed of a suitable tag protein, such as glutathione-S-transferase (GST), protein A, β-galactosidase, and maltose-binding protein (MBP), and the ligand binding domain of PPAR is directly detected in the presence of the substance to be tested.

[0048] According to the method of the invention, for example, screening for an acting agent against PPARγ can be performed. As the ligand for PPARγ, various types of thiazolidinedione derivatives have been identified and prostaglandin, 15d-PGJ_2 (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2), one of arachidonic acid metabolites, is considered to be a true ligand

(Cell, vol. 83, pp. 803-812 and pp. 813-819, 1995). Therefore, upon the identification or screening of an agonist for PPARγ, 15d-PGJ_2 can be used as a positive control. By examining presence or absence of inhibition against ligand-dependent interaction expressed by 15d-PGJ_2, the identification or screening of antagonist to PPARγ can be practiced.

[0049] The agonist for PPARγ is expected as a remedy for treating diabetes having excellent hypoglycemic effect. Since PPARγ is an inducing factor for differentiation of adipocytes, the antagonist to PPARγ is expected to have effect as an anti-obese agent.

[0050] Upon screening PPARγ acting agents, the effect on other subtypes, that is, PPARα or PPARδ (or NUC-1, PPARβ, FAAR) is inspected, whereby medicines having a high selectivity for PPARγ can be selected.

Examples

[0051] In the following, the invention will be explained in more detail by referring to Examples. However, the present invention is not limited thereto.

[0052] In the following examples, unless otherwise specified particularly, each operation was according to the method described in "Molecular Cloning" (written by Sambrook, J., Fritsch, E.F. and Maniatis, T., published by Cold Spring Harbor laboratory Press in 1988) was followed, or when commercially available reagent or kit was used, they were used according to the commercially available specification.

Example 1 Construction of PPARγ acting agent screening system based on ligand-dependent interaction between PPARγ and CBP

(1) Isolation of genes of PPARγ2 and CBP

[0053] cDNA of PPARγ2 was acquired from cDNA library (available from Clontech Co.) by the PCR method. In the PCR, the following primers of SEQ. ID. NOs: 1 and 2 in the Sequence Listing shown below were used. These primers were designed based on the gene sequence of human PPARγ2 described in Accession No. D83233 of gene database, Genbank, and a restriction enzyme recognition site for inserting into yeast expression vector was added to the terminal of the primer. The resulting 1574 base pair fragment had a SmaI recognition site before the start codon and a XhoI recognition site after the stop codon, thus coding for full-length human PPARγ2.

[0054] The cDNA at the N terminus of CBP was obtained by the PCR method from cDNA obtained by reverse transcription reaction from RNA prepared from mouse adipocytes. In the PCR, the primers shown in SEQ. ID. NOs: 3 and 4 in the Sequence Listing shown below were used. These primers were designed based on the gene sequence of human PPARγ2 described in the literature by Chrivia et al. (Nature, vol. 365, 855-859, 1993) to the termini of the primer being added a restriction enzyme recognition site for insertion into yeast expression vector. The resulting 1411 base pair fragment had a BamHI recognition site before the start codon and a BglII recognition site at the C terminus, and coding for the No. 1 to 464 amino acids of mouse CBP.

(2) Construction of expression vector for fused protein comprising ligand binding domain of PPARγ and DNA binding domain of GAL4

[0055] The PPARγ2 gene of 1574 base pair obtained in (1) above was cleaved at the XhoI recognition site designed at the terminus and the BamHI recognition site in the base sequence. The fragments obtained were inserted into the BamHI-SalI site of yeast expression vector pGBT9 (available from Clontech Co., Vector for yeast two hybrid system) containing the gene of the DNA binding domain of transcription factor GAL4 (No. 1 to 147 amino acid residues of GAL4). As a result, plasmid pGBT9-PPARγ2 (Fig. 1A) for expressing a fused protein comprising the portion downstream of the No. 181 amino acid residue of human PPARγ2 (ligand binding domain) and the DNA binding domain of GAL4 was obtained. In Fig. 1A, GAL4 bd stands for a GAL4 DNA binding domain sequence, ^{P}ADH1 stands for alcohol dehydrogenase gene promoter, ^{T}ADH1 stands for an alcohol dehydrogenase gene terminator, Amp^{r} stands for an ampicillin resistant gene, ColE1 ori stands for a collicin E1 replication start point, and 2μ on stands for a 2μ replication start point.

(3) Construction of expression vector for fused protein comprising the N-terminal domain of CBP (PPAR binding domain) and the transcriptional activation domain of GAL4

[0056] The CBP gene of 1411 base pair obtained in (1) above (N-terminal domain) was cleaved at the BamHI recognition site and BglII recognition site designed at the termini. The fragments obtained were inserted into the BamHI-BglII site of yeast expression vector pGAD424 (available from Clontech Co., vector for yeast two hybrid system) containing the gene of the transcriptional activation domain of GAL4 (No. 768 to 881 amino acid residues of GAL4). As a

result, plasmid pGAD424-CBP (Fig. 1B) for expressing a fused protein comprising the portion of the No. 1 to 464 amino acid residues of mouse CBP (N-terminal domain) and the transcriptional activation domain of GAL4 was obtained. In Fig. 1B, GAL4 ad stands for GAL4 transcriptional activation binding domain sequence and others have the same meanings as in Fig. 1A.

(4) Transformation of yeast

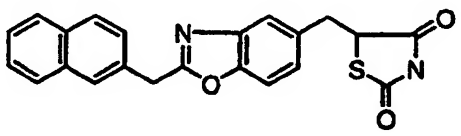
[0057] Using yeast cell strain SFY526 (available from Clontech Co.), the fused protein expression plasmids pGBT9-PPARγ2 and pGAD424-CBP obtained in (2) and (3) above were introduced therein. The cell strain SFY526 (genotype was MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, canr, gal4-542, gal80-538, UPA3::GAL1-lacZ) had incorporated in the chromosome a fused gene of GAL1 and lacZ and is a cell strain having deletion mutation relative to GAL4 gene (Bartel et al., Bio Techniques, vol. 14, pp. 920-924, 1993). The transformation was performed by the lithium acetate method and incubated in a synthetic medium depleted of tryptophan and leucine which are selection markers for the respective plasmids to perform screening to obtain a transformant in which only one of the plasmids was introduced and a transformant in which the both plasmids were introduced.

(5) Detection of ligand-dependent interaction between PPARγ and CBP

[0058] The yeast transformant containing both of the plasmids pGBT9-PPARγ and pGAD424-CBP or the yeast transformant strain containing only one of the plasmids was cultivated in YPD medium (liquid medium). Upon cultivation, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, which is a ligand of PPARγ2 in a living body, diluted with YPD medium was added (or not added). 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (hereinafter abbreviated to as "15d-PGJ_2") used was commercially available (available from CAYMAN CHEMICALS, CO., U.S.A.). The cultivation was performed for 4 to 5 hours. After the cultivation, the yeast cells were recovered by centrifugation and β-galactosidase activity was measured.

[0059] As a result, by the addition of 15d-PGJ_2, an increase in β-galactosidase activity (LacZ gene expression) in yeast containing both of the plasmids PGBT-PPARγ and pGAD424-CBP was observed (Fig. 2A). Such an increase in β-galactosidase activity due to 15d-PGJ_2 was observed dependent on the concentration of 15d-PGJ_2 (Fig. 2B). These were considered to be attributable to the ligand-dependent interaction between PPARγ and CBP due to the presence of the ligand, 15d-PGJ_2. From this result, it revealed that the N-terminal domain of CBP interacts with PPAR. Further, it was considered that in this system, the ligand-dependent interaction between PPARγ and CBP could be detected and measured.

[0060] Next, using as a substance to be tested thiazolidinedione derivative T-174 (chemical name: 5-[[2-(2-naphthalenylmethyl)-5-benzoxazolyl]methyl]-2,4-thiazolidinedione) represented by the following formula:



its effect on PPARγ was examined.

[0061] In the same manner as mentioned above, the yeast transformant containing both of the plasmids pGBT9-PPARγ and pGAD424-CBP or a yeast transformant containing only one of the plasmids was cultivated. However, upon cultivation, T-174 was added as a substance to be tested instead of 15d-PGJ_2 in the medium. T-174 used was synthesized by a method similar to that described in Japanese Provisional Patent Publication No. 56675/1989 (Example 49).

[0062] As a result, an increase in β-galactosidase activity was observed only in the yeast containing both of the plasmids pGBT-PPARγ and pGAD424-CBP (Fig. 3A). Its effect was dependent on the concentration of 15d-PGJ_2 (Fig. 3B). Thus, the ligand-dependent interaction between PPARγ and CBP was detected due to the presence of T-174, so that T-174 was identified as an agonist acting as a ligand to PPARγ.

[0063] T-174 is known to have a hypoglycemic effect in a disease model of mouse (KK-Ay mouse) (Japanese Provisional Patent Publications No. 56675/1989 and No. 167225/1990). Although its acting point was unclear, the above results indicate that the acting target molecule of T-174 is PPARγ.

Example 2 Construction of PPARγ acting agent screening system based on the ligand-dependent interaction between PPARγ and SRC-1

[0064] cDNA of the full domain of SRC-1 is obtained by the PCR method from the cDNA library prepared from human adipose tissue. The primers are designed based on the gene sequence of human SRC-1 described in the literature of Onate et al. (Science, vol. 270, pp. 1354-1357, 1995) and to the terminus of the primer is added a restriction enzyme recognition site for insertion of yeast expression vector.

[0065] This is used instead of the cDNA of CBP and, in the same manner as in Example 1 (2) and (3), the cDNA of PPARγ2 is inserted into the yeast expression vector pGBT9 and the cDNA of SRC-1 is inserted into the yeast expression vector pGAD424, respectively, whereby an expression vector for a fused protein comprising the ligand binding domain of PPARγ and the DNA binding domain of GAL4, and an expression vector for a fused protein comprising the full domain of SRC-1 and the transcriptional activation domain of GAL4 are constructed.

[0066] The resultant two kinds of fused protein expression plasmid are introduced into yeast cell strain SFY526 of which a fused gene of GAL1 and lacZ is incorporated in its chromosome and having a deletion mutation regarding GAL4 gene in the same manner as in Example (4) above.

[0067] Using the obtained transformed strain, the ligand-dependent interaction between PPARγ and SRC-1 is detected in the same manner as in Example (5) above.

INDUSTRIAL APPLICABILITY

[0068] The conventional identification method for PPAR acting agent detecting the transcriptional activation ability of PPAR in the cells accepts participation of a coactivator and RXR which are intrinsic to the cells. The method of this invention is tree from this participation, so that only the effect of the substance to be tested to PPAR can be detected with accuracy. Also, the method of the invention does not have to use mammalian cells and can use yeast cells as well, so that cultivation operations can be performed with ease and quickly. Further, there is no need for using radioisotope-labeled compound to be tested or protein and hence the method is safe and simple.

[0069] According to the method of the invention, since it is possible to treat a number of substances to be tested simultaneously with sufficient sensitivity and quantitativeness, the identification and screening of agonist for and antagonist to PPAR can be performed efficiently.

SEQUENCE LISTING

<110> TANABE SEIYAKU CO. LTD.
<110> TANIGUCHI Tomoyasu
<110> MIZUKAMI Junko
<120> Identification or screening method of agonist or antagonist of PPAR
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Claims

1. A method for identifying or screening an agonist for or antagonist to a peroxisome proliferator-activated receptor (PPAR), which comprises allowing a test cell and a substance to be tested to coexist, and detecting a change in a ligand-dependent interaction between the PPAR and a coactivator in the test cells due to the substance to be tested by measuring the expression of a reporter gene as an index.

2. The method according to Claim 1, wherein the coactivator and PPAR in the test cell are derived from an extrinsic gene.

3. The method according to Claim 1, wherein the test cells contain

   (i) a first fused gene coding for a first fused protein comprising at least ligand binding domain of PPAR and a first domain of a transcription factor, wherein the first domain of said transcription factor being a DNA binding domain or a transcriptional activation domain;
   (ii) a second fused gene coding for a second fused protein comprising at least PPAR binding domain of a coactivator which interacts with the PPAR and a second domain of the transcription factor, wherein the second domain of said transcription factor is a transcriptional activation domain when the first domain of the transcription factor is a DNA binding domain or is a DNA binding domain when the first domain of the transcription factor is a transcriptional activation domain, and
   (iii) a response element to which the DNA binding domain of said transcription factor can bind and a reporter gene linked thereto.

4. The method according to Claim 1, wherein the test cells are yeast cells.

5. The method according to Claim 1, wherein the PPAR is PPARγ.

6. The method according to Claim 5, wherein the PPARγ is derived from human.

7. The method according to claim 1, wherein the coactivator is selected from CBP and SRC-1.

8. The method according to Claim 1, wherein the coactivator is CBP.

9. The method according to Claim 3, wherein the transcription factor is GAL4 protein of yeast.

10. A method for identifying or screening an agonist for or antagonist to PPAR, characterized by detecting a ligand-dependent interaction between PPAR and CBP, and measuring the effect of a substance to be tested on said interaction.

11. The method according to Claim 10, wherein the interaction between a ligand binding domain of the PPAR and a nuclear receptor binding domain of the CBP is detected.

FIG. 1A

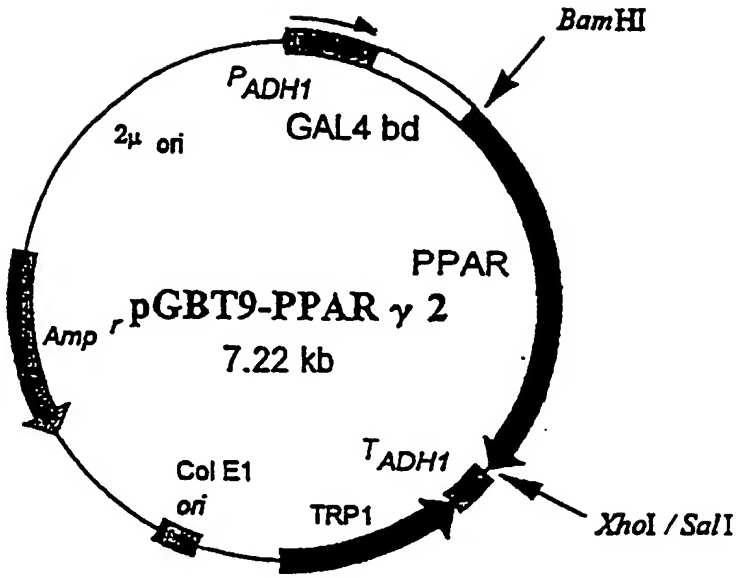


FIG. 1B

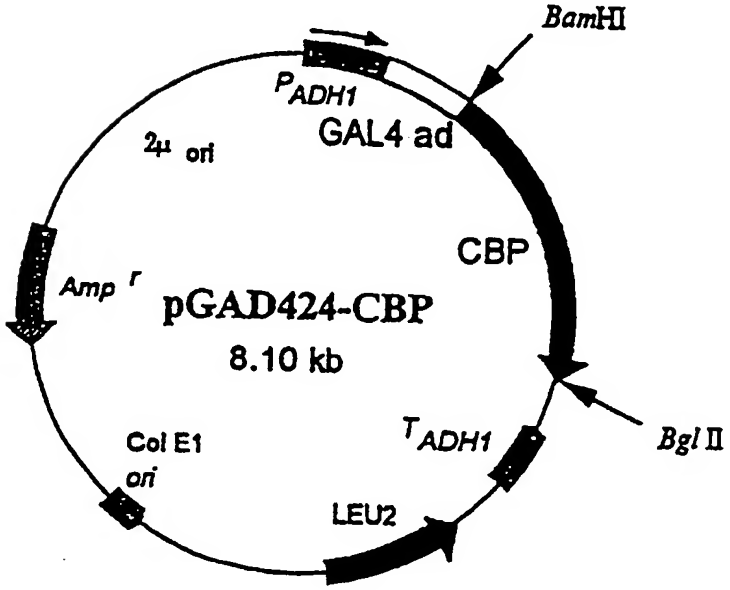


FIG. 2A

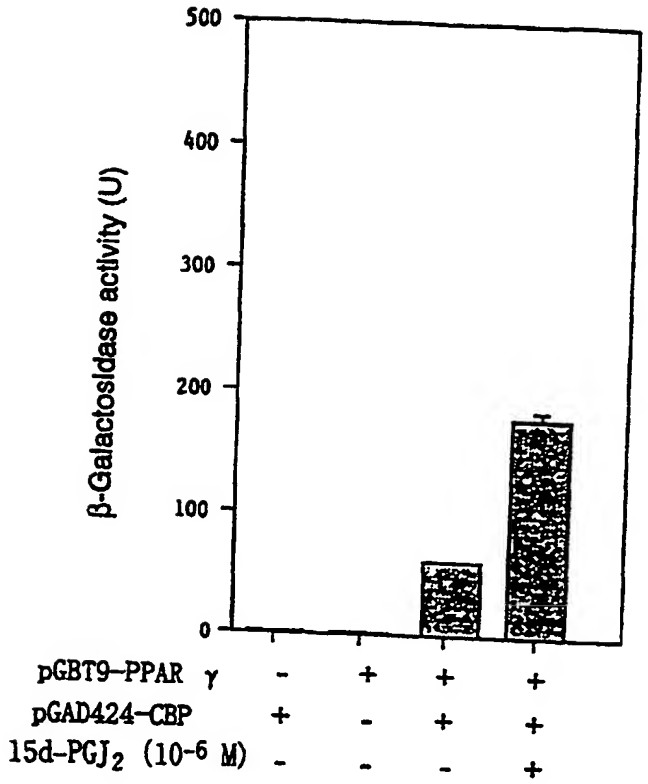


FIG. 2B

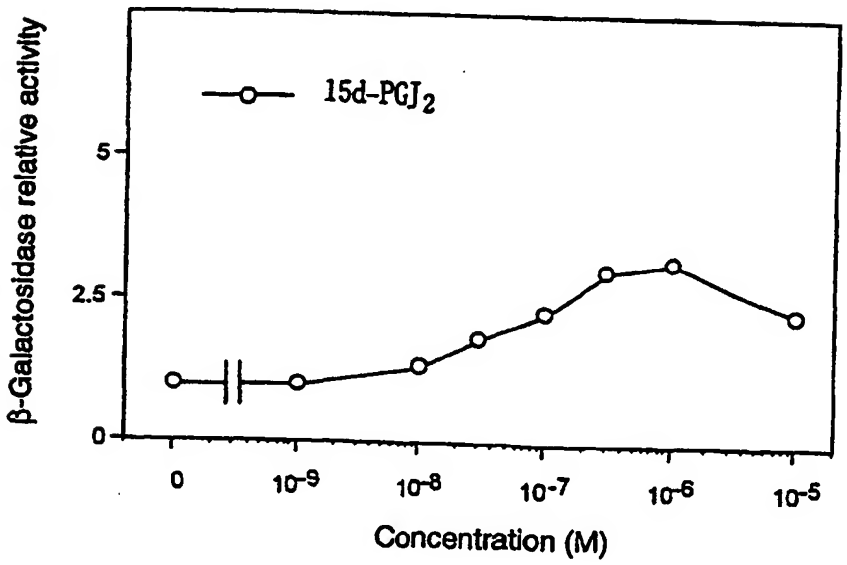


FIG. 3A

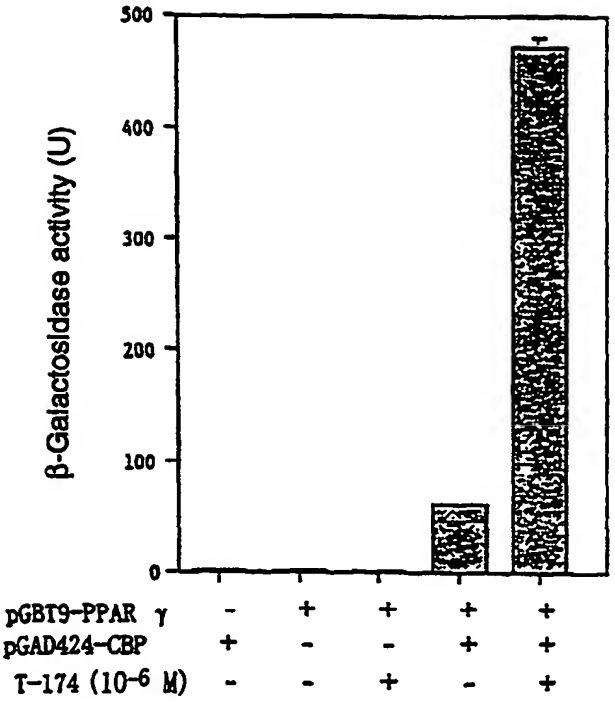
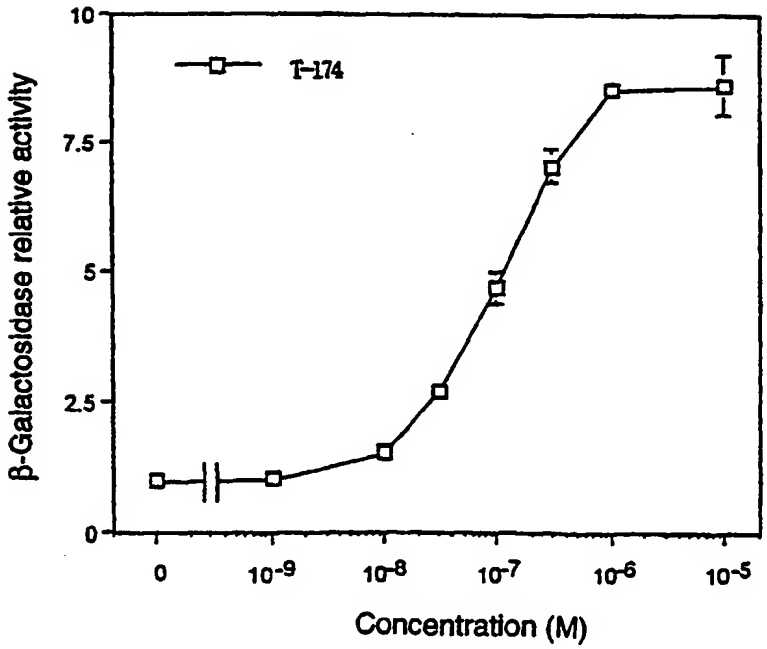


FIG. 3B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/03734

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C12Q1/68, C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C12Q1/00-3/00, C12N15/11-15/62

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS (DIALOG), WPI (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	Molecular Endocrinology, Volume 11, Number 6, issued June 1997, Grigorios Krey et al., "Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay", pages 779-791	1-7, 9 8, 10, 11
Y	US, 5,283,173, A (The Research Foundation of State University of New York), 1 February, 1994 (01. 02. 94) (Family: none)	1-7, 9
P, X	Biochemical Biophysical Research Communications, Volume 240, Number 1, issued 7 November 1997, Junko Mizukami and Tomoyasu Taniguchi, "The Antidiabetic Agent Thiazolidinedione Stimulates the Interaction between PPAR γ and CBP", pages 61-64	1-11
P, X	Molecular Endocrinology, Volume 12, Number 6, issued June 1998, Eckardt Treuter et al., "A Regulatory Role for RIP140 in Nuclear Receptor Activation", pages 864-881	1-7, 9

[x] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November, 1998 (17. 11. 98)	1 December, 1998 (01. 12. 98)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/03734

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	The Journal of Biological Chemistry, Volume 272, Number 41, issued 10 October 1997, Yijin Zhu et al., "Isolation and Characterization of PBP, a Protein That Interacts with Peroxisome Proliferator-activated Receptor", pages 25500-25506	1-7, 9

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/03734

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The group of inventions as set forth in claims 1 to 9 relates to methods for identifying or screening agonists for or antagonists to a peroxisome proliferator-activated receptor (PPAR) by detecting a change in the ligand-dependent interaction between the PPAR and a transcriptional coupling factor due to the object by measuring as an indication the expression of a reporter gene which is a specific means for measurement, while the group of inventions as set forth in claims 10 and 11 relates to methods for identifying or screening agonists for or antagonists to a PPAR by measuring a change in the ligand-dependent interaction between the PPAR and CBP which is a transcriptional coupling factor due to the object.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/03734

Continuation of Box No. II of continuation of first sheet (1)

Such being the case, these two groups of inventions are not considered as relating to a group of inventions so linked as to form a single general inventive concept.

Form PCT/ISA/210 (extra sheet) (July 1992)